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(54) Title: PROCESS FOR PREPARING STARTER CULTURES OF LACTIC ACID BACTERIA

(57) Abstract

The present invention relates to a process for preparing lactic acid bacterial starter cultures, which comprises: culturing at least one strain of lactic acid bacteria under aeration and in an appropriate nutrient medium, in which at least one porphyrin compound is present or is added; harvesting the bacteria at the end of the said culture. The invention also relates to the use of lactic acid bacterial starter cultures obtained according to said process for preparing a fermented product.

PROCESS FOR PREPARING STARTER CULTURES OF LACTIC ACID BACTERIA

The present invention relates to a novel process for preparing lactic acid bacterial starter cultures that exhibit preservation and acidification properties superior to those of conventionally used starter cultures.

The preparation of fermented products begins with the inoculation of a food substance with a starter culture that consists of one or more bacterial strains having the desired characteristics for producing the final product.

Ready-to-use starter cultures are marketed in the form of bacterial preparations that are generally frozen or lyophilized. In order to ensure that the fermentation starts off well, the bacteria comprising the starter culture should first be cultured, harvested, packaged and stored under conditions that are optimal for their growth and also for their survival. Furthermore, in order to obtain a final product of constant quality, the conditions for preparing the starter culture should be reproducible.

However, different stress conditions that may occur during the different steps of preparing starter cultures can result in altered growth and/or survival of the bacteria.

First of all, when lactic acid bacteria are cultured, the medium becomes acidified as a natural consequence of bacterial growth. This acidification arrests cell division when the pH of the medium reaches a value of around 4.5, and also decreases cell viability.

In addition, aside from lactic acid, lactic acid bacteria also produce various other antibacterial substance during growth, such as nisin, bacteriocins, various organic acids and diacetyl. Since these substances are more active on certain contaminating bacteria that may be present in the culture, than on the lactic acid bacteria themselves, their production in the medium initially favours growth of the latter. However, accumulation of these substances during the culture can also become detrimental to the survival of the lactic acid bacteria.

KANEKO et al. [Appl. Environ. Microbiol., 56:9, 2644-2649 (1990)], describe the culturing of a strain of lactic acid bacteria which possesses a high NADH oxidase activity and a high diacetyl synthase activity (strain 3022 of *L. lactis* subsp. *lactis* biovar *diacetylactis*), under aerobic conditions and in the presence of hemin and/or Cu^{2+}. They observe a substantial increase in the production of diacetyl and acetoin. In the course of the culture, they also observe a rise in pH, following an initial drop, the overall result being a decline in the acidification of the culture

In the above documents no information is provided with regard to the possible effects of these culture conditions on the viability of the bacteria and their ability to restart growth if they are once again placed under conventional fermentation conditions.

The present invention solves these problems through a novel process for preparing starter cultures comprised of lactic acid bacteria resulting in an improved yield, and increased survival of said bacteria, and in an improvement of acidifying properties of said starter cultures.

Thus, the inventors observed that the addition of a porphyrin compound to the culture medium of lactic acid bacteria that are cultured under aeration, not only increased the yield of the cultures but also the viability of the bacteria and their resistance to the various stress conditions that can occur during the culture and during the packaging and storage of the starter cultures.

The present invention relates to the use of a porphyrin compound in association with an aerobic culture for increasing the survival of lactic acid bacteria at the end of the said culture. In particular, the present invention relates to a process for preparing a lactic acid bacterial starter culture, for which the process comprises:

- culturing at least one strain of lactic acid bacteria under aeration and in an appropriate nutrient medium in which at least one porphyrin compound is present or is added;

- harvesting the bacteria at the end of the said culture.

"Lactic acid bacteria" refers to a group of bacteria that belong to various genera and that are used in processes for fermenting food products. This group is principally composed of bacteria in which the main product of carbohydrate metabolism is lactic acid. However, bacteria that produce low quantities of lactic acid (*Leuconostoc* and propionic acid bacteria) are included in this list due to their use in fermentation processes. In general, the lactic acid bacteria concerned are those belonging to the genera *Lactococcus, Lactobacillus, Leuconostoc, Propionibacterium,* and *Bifidobacterium*, or *Streptococcus salivarius*.

"Lactic acid bacterial starter culture" refers to any preparation that is intended for inoculating a medium to be fermented and that comprises a bacterial strain or a mixture of strains belonging to one of the above-mentioned genera; such a starter culture can also comprise, or consist of, strains of mutant bacteria and/or strains of recombinant bacteria that are derived from bacteria belonging to the above-mentioned genera.

2.5 to approximately 100, preferably at a concentration of approximately 5 to approximately 40, micromoles per litre of culture medium.

According to another preferred embodiment of the present invention, the culture is aerated so as to maintain, during the entire duration of the culture, an oxygen content which is equal to at least 5 millimoles per litre of culture medium, preferably from 8 to 45 millimoles per litre of culture medium. Aeration can be effected by any means known by one skilled in the Art, for example by shaking or stirring the culture medium, or by passing a gaseous mixture containing oxygen such as air, into the culture medium.

When the present invention is implemented, a bacterial biomass is obtained which is more substantial than that obtained when starter cultures are prepared by conventional methods. Furthermore, there is a greater percentage of viable bacteria that are metabolically active in the bacterial population.

Despite this substantial bacterial growth, the medium is found to be only weakly acidified; the pH decreases less rapidly than in the case of a culture that is not aerated and in which there is no porphyrin compound, and this pH generally stabilizes at a value varying between approximately 5 and approximately 7. When the present invention is implemented, the decrease in the pH is regular; the pH is not found to fall and then rise again, contrary to what KANEKO et al. observed in the case of the *L. lactis* strain 3022.

In the process of the invention, the quantity of glucose in the culture medium converted into lactic acid is less than approximately 40% by weight of the total quantity of glucose initially present. The quantity usually varies between approximately 5% and approximately 30% of the total quantity of glucose that is initially present.

The bacteria are harvested when it is considered that the bacterial population has reached a sufficiently high level. In the methods of the prior art, it is difficult to control the time of harvesting precisely; thus, while it is necessary to have achieved a sufficiently high bacterial population, it is also a requirement that the said bacterial population should still contain a maximum of viable bacteria. On the other hand, use of the process according to the invention makes it possible, at one and the same time, to increase the growth of the bacteria and their viability, thereby providing a much wider latitude for carrying out the harvesting.

For example, it is possible to harvest the bacteria from 5 to 24 hours, advantageously from 7 to 13 hours, after the start of the culture.

The cryoprotectant employed is advantageously alginate, glycerol, glycine betaine, skimmed milk, trehalose or sucrose.

The present invention also encompasses the lactic acid bacterial starter cultures that can be obtained by the process according to the invention. These starter cultures can comprise one or more species of lactic acid bacteria and/or one or more strains of one and the same species, with all or some of the said species or the said strains having been cultured in accordance with the invention. Several different species or several different strains may be cultured simultaneously (when their optimal growth conditions are compatible) or else cultured separately and combined after harvesting.

The lactic acid bacterial starter cultures which have been obtained in accordance with the invention can be used to inoculate a medium to be fermented, in particular within the context of transforming raw materials of animal or plant origin, for example for producing food products, such as fermented dairy products, or for producing molecules of interest in a fermenter.

Upon inoculation of a starter culture prepared according to this invention, and using any of a variety of lactococcal strains and subspecies, one observes that the culture starts growth, and acidifies rapidly, even after the starter culture has been stored for a long period, which demonstrates that a substantial proportion of bacteria in the starter culture are viable and metabolically active, and furthermore that these bacteria, which are derived from cultures carried out in the presence of a porphyrin compounds and under aeration, are able to readapt rapidly to the usual conditions of lactic acid fermentation.

The invention will be illustrated in more detail in the description that follows and that refers to non-limiting examples of the implementation of the process of the invention.

I) Figure legends

- Figure 1: Growth and survival of *Lactococcus lactis*, which is cultured and stored at 30°C.

This figure compares the growth and survival curves of lactic acid bacteria (in this case *Lactococcus lactis*) that are cultured at 30°C with or without hemin and aeration according to the process of the invention. The number of viable cells is expressed as a function of time (number of days after inoculation, which is time zero), with the storage temperature of the bacteria being 30°C. The curve with the circles ( ) corresponds to bacteria cultivated by the method of the prior art (without hemin), whereas that with the squares (■) corresponds to the culture

Lactococcus lactis subsp. *cremoris* strain MG1363 in the form of a 1/100 or 1/1000 dilution of a saturated culture (prepared at 30°C with no shaking). The inoculated medium contained or not hemin at a final concentration of 10 µg/ml (a 0.5 mg/ml stock solution is prepared by dissolving 100 mg of hemin in 2 ml of 5N NaOH, to which 198 ml of water is then added; the solution is autoclaved at 120°C for 20 minutes). For bacteria grown in medium containing hemin, cultures were maintained at 30°C with shaking (250 rotations per minute) in order to ensure oxygenation. Control cultures, without addition of hemin and without shaking, were grown at 30°C in parallel. After 24 h of growth, aliquots are removed to measure the optical density at 600 nm ($OD_{600\ nm}$), the number of viable bacteria, the final pH and the concentration of lactic acid in the medium. The results are shown in Table I.

These results show that a greater biomass is achieved when the bacteria are cultured in the presence of hemin and oxygen. This increase is demonstrated by the higher optical density values. Furthermore, a higher number of viable cells is observed when the cells are cultured in the presence of hemin and oxygen. It may also be noted that, when the cells are cultured in the presence of hemin and oxygen, the pH does not vary greatly and remains stable around a value of approximately 6.1 whatever the concentration of glucose. In contrast, in the case of cells cultured under conventional conditions, the pH is markedly lower at a glucose concentration of 1% (pH 4.5) than when the glucose concentration is at 0,5% (pH 5.7). It is also observed that the production of lactic acid by cells cultured in the presence of hemin and oxygen is low and is always less than 10% of the quantity of sugar added, whereas, in the case of the control cultures, approximately 80% of the added glucose is converted to lactic acid.

2) Culturing in the presence of protoporphyrin IX

A laboratory medium (M17 supplemented with 1% glucose) is inoculated with the *Lactococcus lactis* subsp. *cremoris* strain MG1363 in the form of a 1/1000 dilution of a saturated culture (prepared at 30°C with no shaking). The inoculated medium contained or not protoporphyrin IX at a final concentration of 10 µg/ml (a 0,5 mg/ml stock solution is prepared by adding 100 mg of protoporphyin IX to 2 ml of 5N NaOH, to which 198 ml of water is then added; the solution is autoclaved at 120°C for 20 minutes). For bacteria grown in medium containing protoporphyrin IX, cultures were maintained at 30°C with shaking (250 rotations per minute) in order to ensure oxygenation, or without shaking as a control. Two other control cultures, without protoporphyin IX or without shaking, were grown at 30°C in parallel. After 24 h of growth, aliquots are removed to measure the optical density at 600 nm ($OD_{600\ nm}$), the number of viable bacteria, the final pH and the concentration of lactate in the medium. The results are compiled in Table II.

chlorophyllin at a concentration of 100 µg/ml is filtered and then added to the culture medium so as to obtain a final concentration of 10 µg/ml.

b) by incubating a concentrated solution of chlorophyllin for 24 h in M17 at a pH of between 3 and 5 (acidified with HCl) and at a temperature of 4°C, 30°C or 60°C. The pH is then readjusted to 7. The chlorophyllin solution, at a concentration of 100 µg/ml, is subsequently filtered and then added to the culture medium in order to obtain a final concentration of 10 µg/ml.

c) by incubating a concentrated solution of chlorophyllin for 24 h in M17 at a pH of 4.5 (acidification by adding lactate) and at a temperature of 4°C, 30°C or 60°C. The pH is then readjusted to 7. The chlorophyllin solution, at a concentration of 100 µg/ml, is subsequently filtered and then added to the culture medium in order to obtain a final concentration of 10 µg/ml.

For bacteria grown in medium containing chlorophyllin, cultures are placed at 30°C, with shaking in order to oxygenate the cultures (250 rotations per minute). Control cultures, to which no chlorophyllin is added and which are not shaken, are grown in parallel. After 24 h of growth, aliquots are removed for measuring the optical density at 600 nm ($OD_{600\ nm}$) and the final pH.

In all the cultures carried out using preparations a), b) or c), and under aeration, an $OD_{600\ nm}$ which is higher than that of the control cultures by from 0.3 to 0.8 units, and a pH which is higher than that of the control cultures by from 0.3 to 0.8 units, are observed, depending on the samples concerned. No significant variation from the control is observed when the cultures carried out in the presence of chlorophyllin are not shaken.

EXAMPLE 2: EFFECT OF CULTURING IN THE PRESENCE OF OXYGEN AND OF A PORPHYRIN DERIVATIVE, ON THE SURVIVAL OF THE LACTIC ACID BACTERIA DURING STORAGE AT 30°C

A laboratory medium, i.e. M17 supplemented with 1% of glucose, is inoculated with *Lactococcus lactis* subsp. *cremoris* strain MG1363 in the form of a 1/1000 dilution of a saturated culture. The cultures are then divided into two equal parts, and hemin is added to one of these two cultures to give a final concentration of 10 µg/ml. The control culture, which does not contain any hemin, is incubated at 30°C without shaking, whereas that containing hemin is incubated at 30°C with shaking (250 rotations per minute) in order to oxygenate it. Aliquots of the two cultures are removed regularly during the exponential growth phase in order to monitor viability, rate of growth and pH. After 24 h of growth, the cultures are placed (stored) in a 30°C

Table IV

Number of days after culture	Number of cells per ml							
	0	1	2	3	7	8	9	10
1% glucose M17 + hemin and aeration	4.3×10^{9}	4.2×10^{9}	3.8×10^{9}	3.8×10^{9}	4.2×10^{9}	2.8×10^{9}	2.2×10^{9}	9.6×10^{8}
1% glucose M17 + protoporphyrin IX and aeration	6.4×10^{9}	6.2×10^{9}	5.6×10^{9}	5.4×10^{9}	4.0×10^{9}	2.8×10^{9}	1.9×10^{9}	3.9×10^{8}
1% glucose M17	1.6×10^{9}	1.3×10^{9}	5.3×10^{8}	3.5×10^{8}	2.0×10^{6}	2.0×10^{4}	2.1×10^{3}	1.9×10^{3}

Number of days after culture	Number of cells per ml							
	13	15	22	28	36	43	50	57
1% glucose M17 + hemin and aeration	5.0×10^{8}	3.2×10^{8}	3.0×10^{8}	3.0×10^{8}	2.9×10^{8}	2.2×10^{8}	1.8×10^{8}	2.0×10^{8}
1% glucose M17 + protoporphyrin IX and aeration	1.7×10^{8}	6.0×10^{7}	5.9×10^{7}	5.4×10^{7}	5.4×10^{7}	5.3×10^{7}	4.8×10^{7}	5.3×10^{7}
1% glucose M17	4.0×10^{2}	2.4×10^{1}	$<2.0\times10^{0}$	$<2.0\times10^{0}$	$<2.0\times10^{0}$	$<2.0\times10^{0}$	$<2.0\times10^{0}$	$<2.0\times10^{0}$

hemin and with a flow of air. When growth is terminated, the cultures are centrifuged and the pellets are frozen in liquid nitrogen and then stored at -80°C.

The acidification properties of the starter cultures prepared by conventional means a) or by the process of the invention b) are measured at day 0 and after 5, 8, 13, 21 and 30 days at -80°C. The growth medium, 9.5% reconstituted skimmed milk, is inoculated with 0.01% (w/v) of starter culture 'a)' or 'b)', as above, and the fermentation is carried out at 30°C without shaking; the pH is measured continuously.

The results are shown in Figure 3 (a-f). These results demonstrate that:

- neither of the 2 starter cultures loses its properties during storage at -80°C;

- from the time of its preparation, and during the entire storage period, starter culture b) according to the invention exhibits properties significantly superior to those of starter culture a), which is obtained using conventional methods. In particular, starter culture b) reaches a predetermined pH 30 to 40 minutes before starter culture a), while showing the same acidification kinetics.

It appears therefore, that the addition of hemin combined with culturing under aerobic conditions significantly improves the production of a lactic acid bacterial starter culture, and in particular the acidification performance of the said starter culture.

13) Use of a lactic starter culture according to Claim 10 for preparing a fermented product.

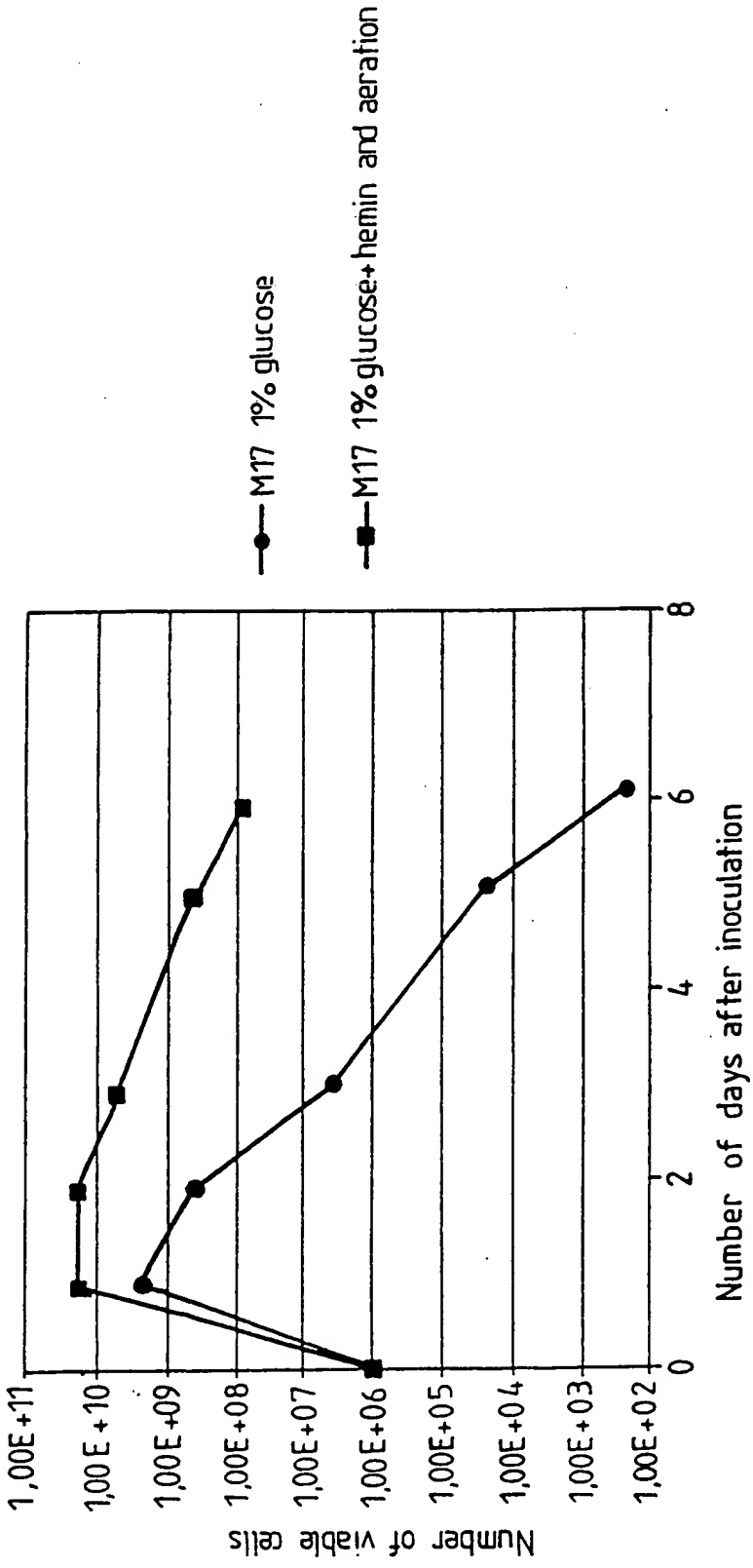
M17 1% glucose
M17 1% glucose+hemin and aeration
Number of viable cells
1,00E+11
1,00E+10
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
0
2
4
6
8
Number of days after inoculation

FIG.1

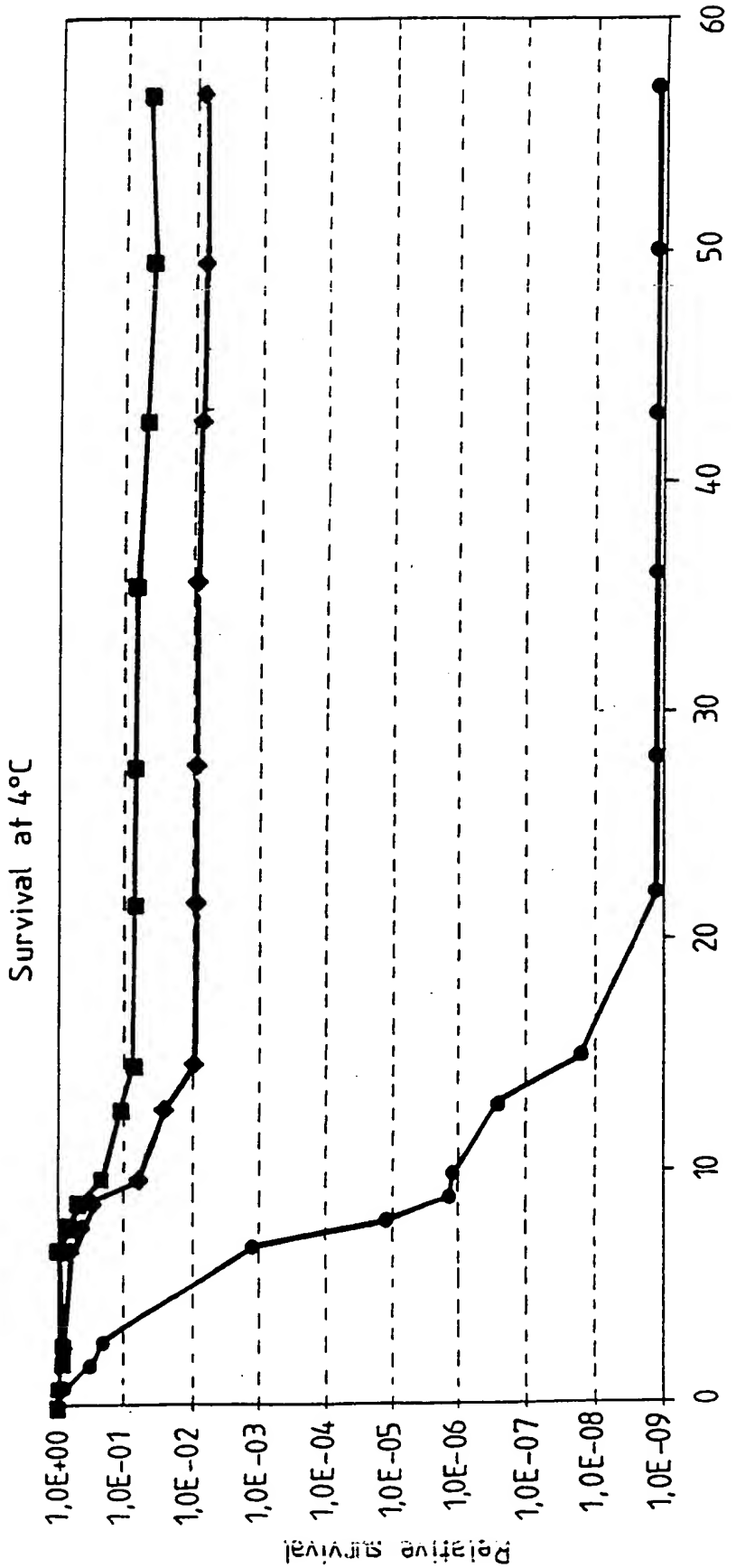
Survival at 4°C
Relative survival
1,0E+00
1,0E-01
1,0E-02
1,0E-03
1,0E-04
1,0E-05
1,0E-06
1,0E-07
1,0E-08
1,0E-09
0
10
20
30
40
50
60
Number of days at 4°C

FIG.2

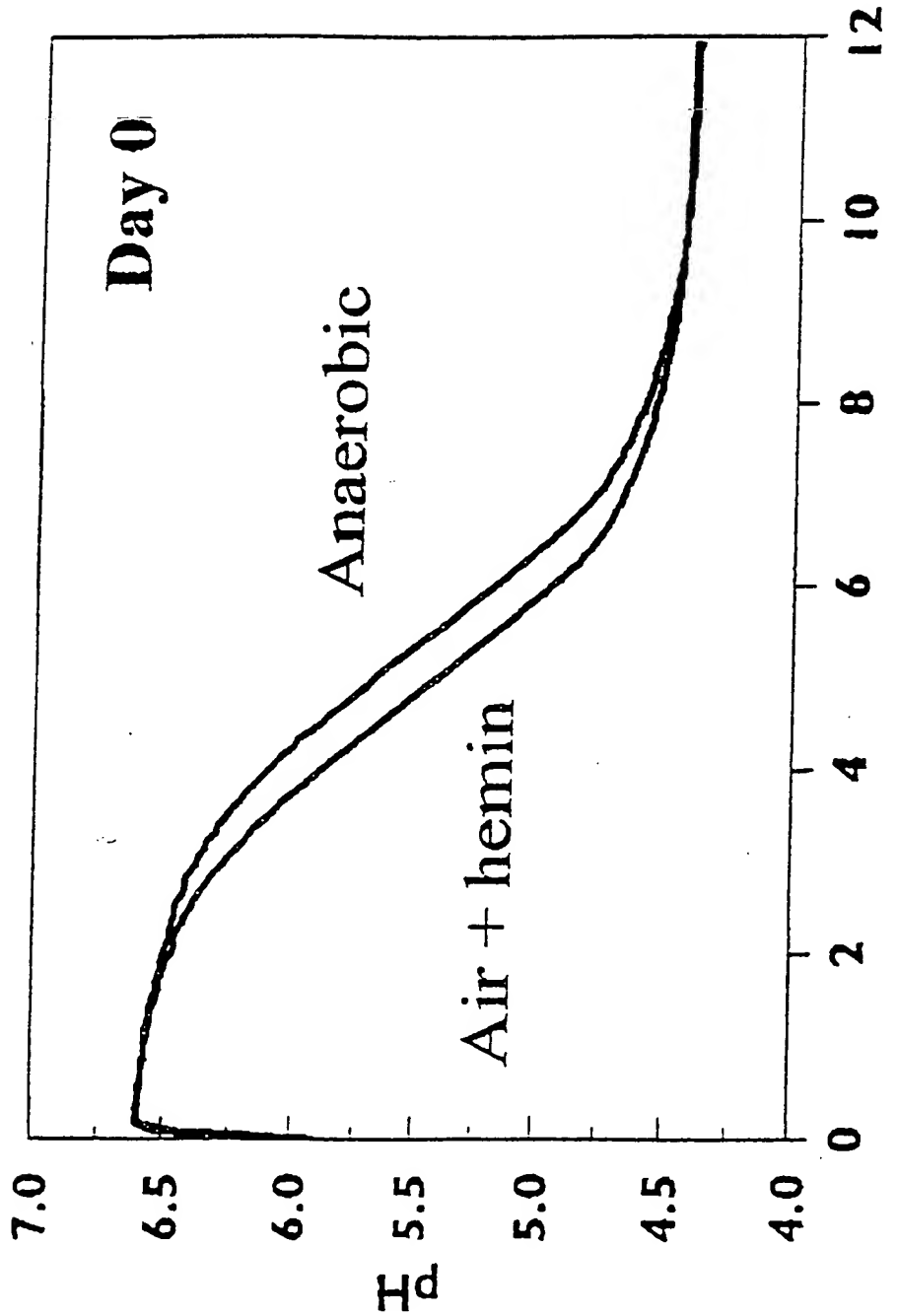
Day 0
Anaerobic
Air + hemin
pH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
2
4
6
8
10
12
Time [h]

Figure 3a

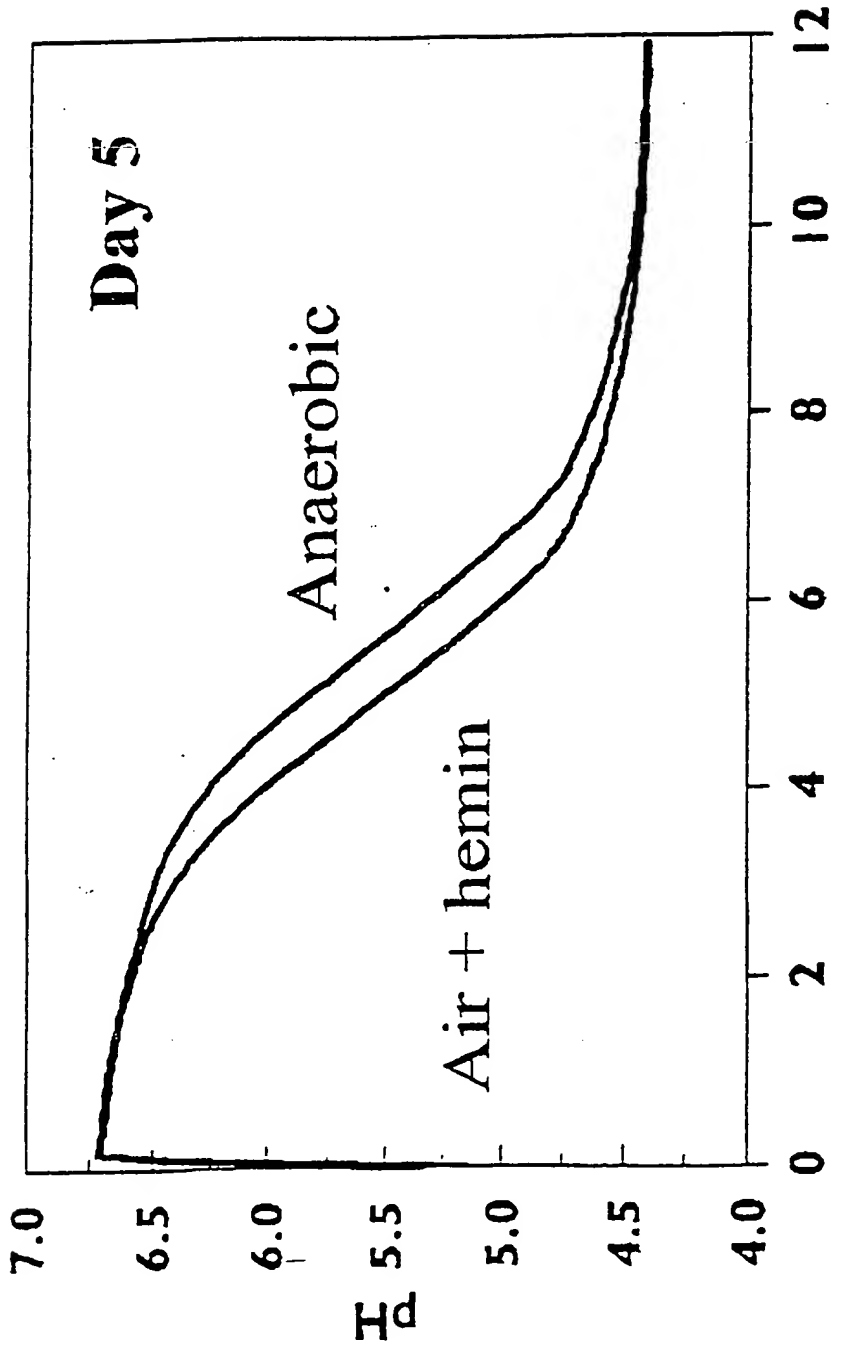
Day 5
Anaerobic
Air + hemin
pd
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
2
4
6
8
10
12
Time [h]

Figure 3b

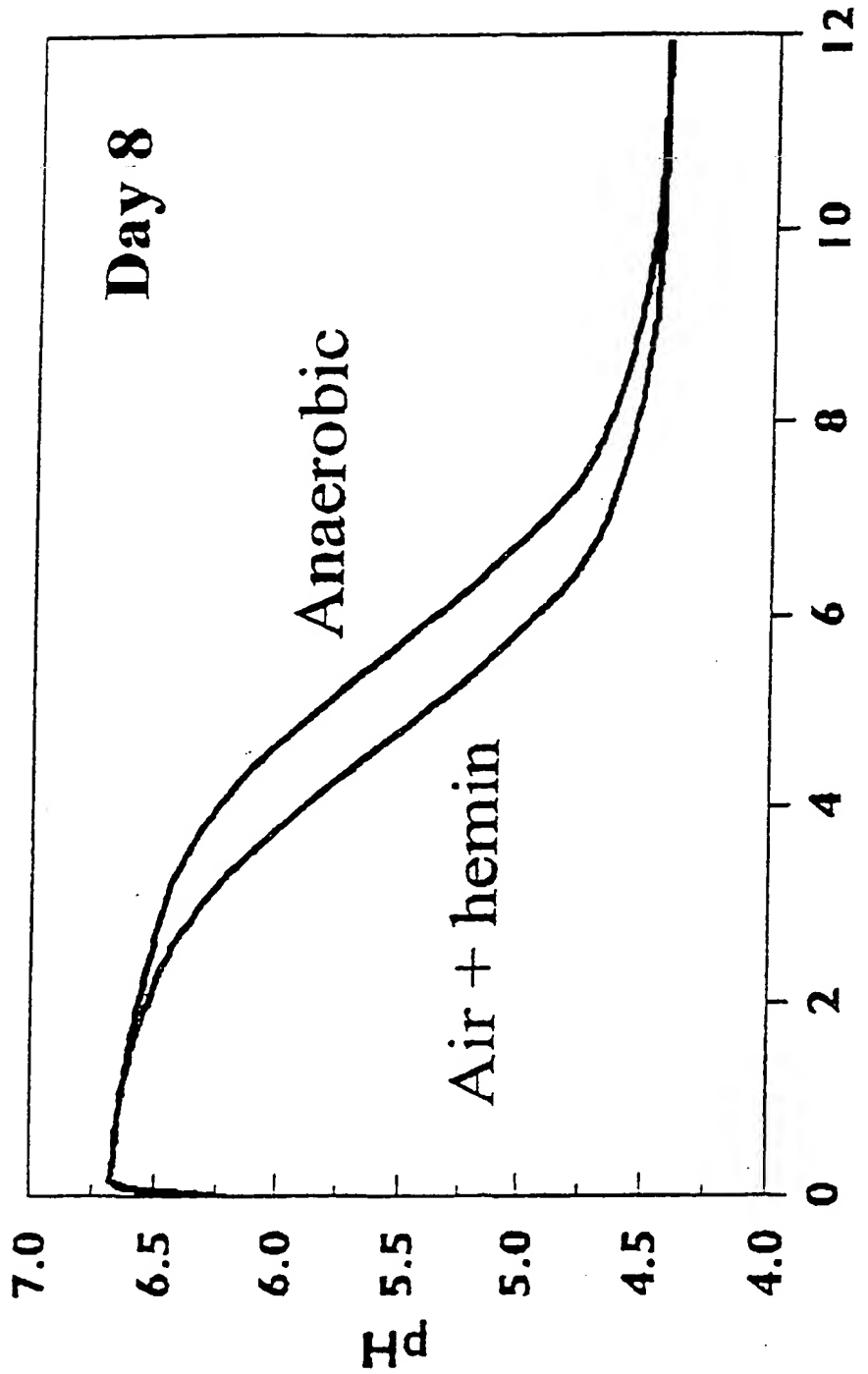
Day 8
Anaerobic
Air + hemin
Time [h]
pH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
2
4
6
8
10
12

Figure 3c

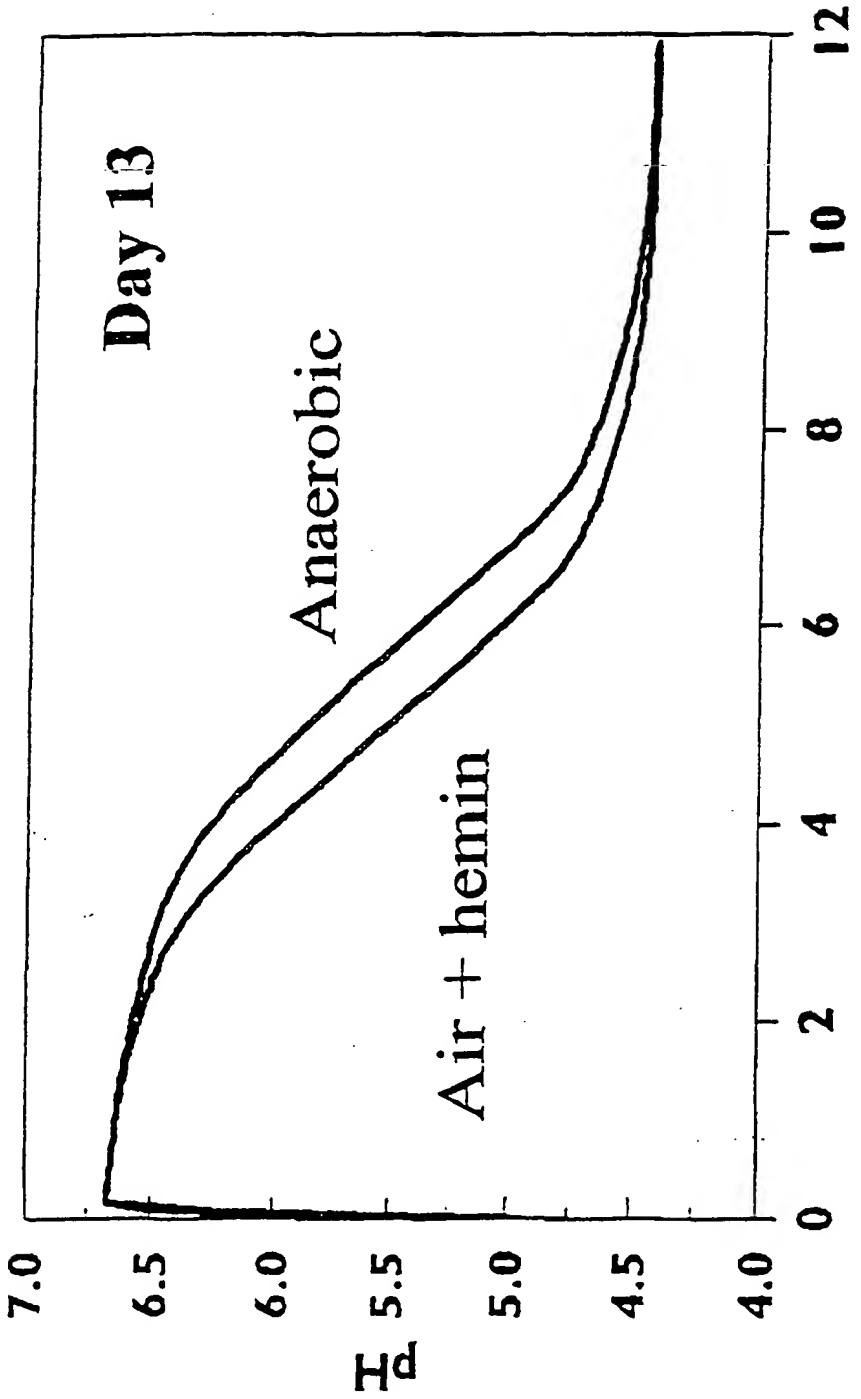
Day 13
Anaerobic
Air + hemin
pH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
2
4
6
8
10
12
Time [h]

Figure 3d

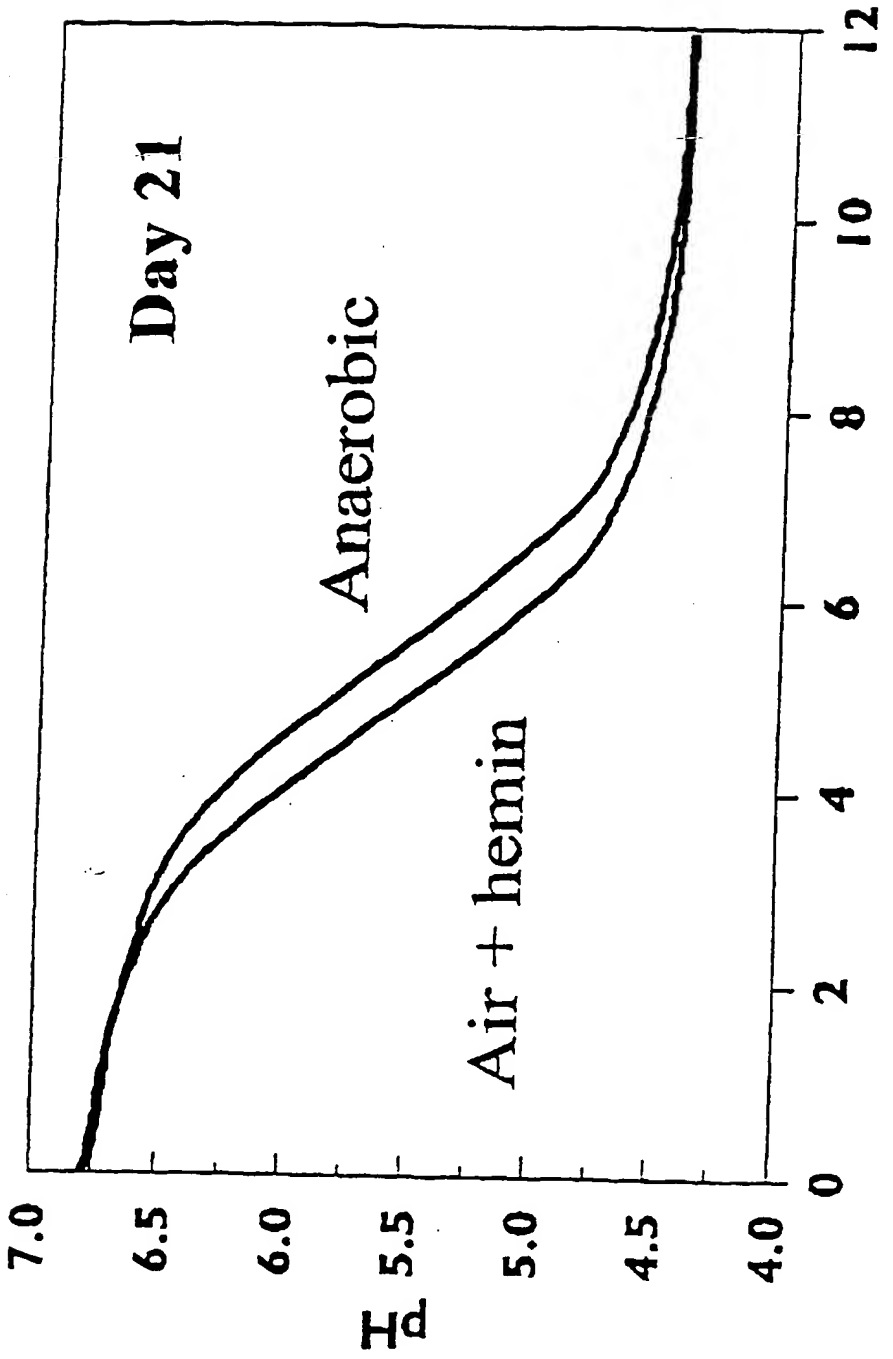
Day 21
Anaerobic
Air + hemin
pH
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
2
4
6
8
10
12
Time [h]

Figure 3e

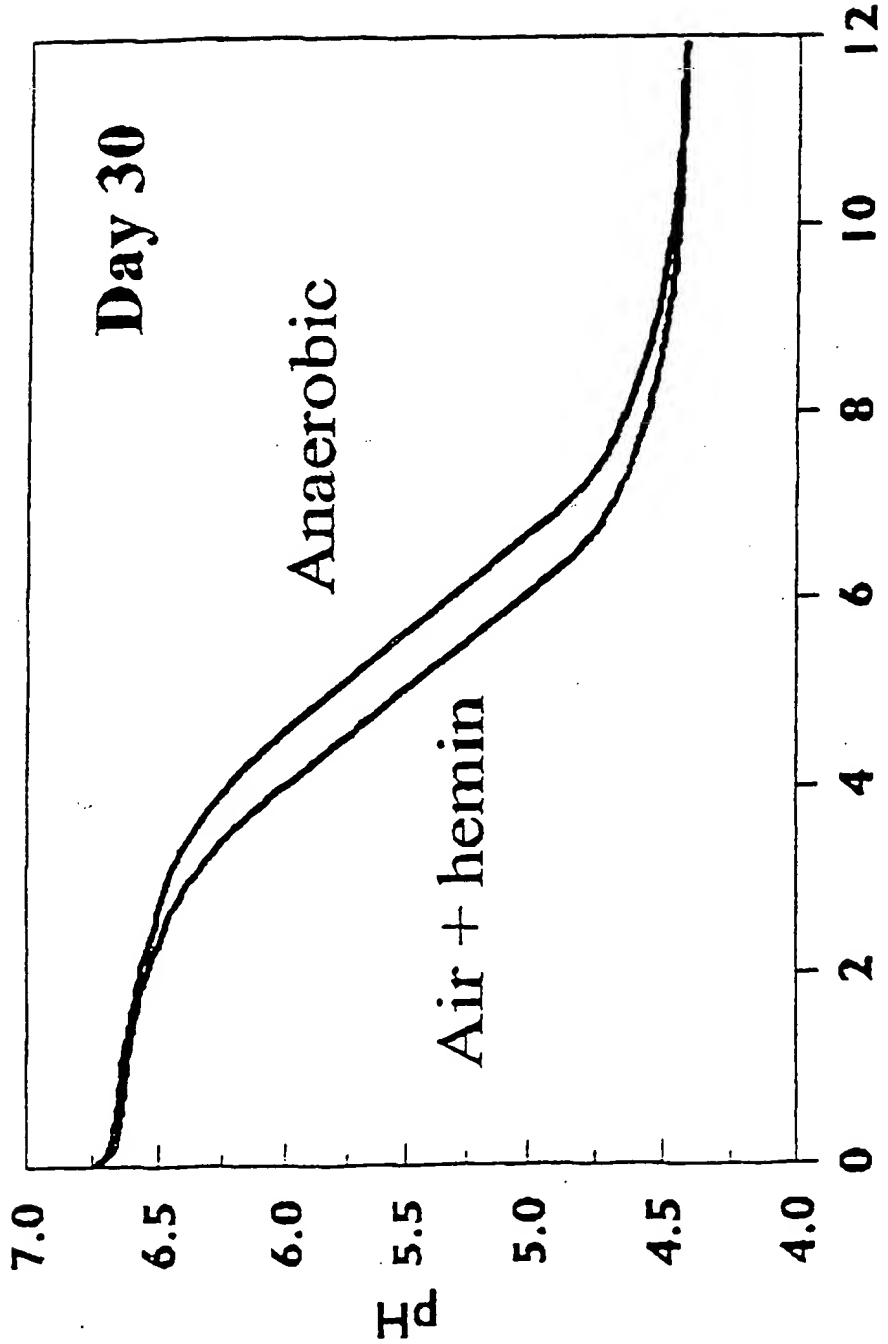
Day 30
Anaerobic
Air + hemin
Time [h]
0
2
4
6
8
10
12
7.0
6.5
6.0
5.5
5.0
4.5
4.0
pH

Figure 3f

INTERNATIONAL SEARCH REPORT

Inte · ttional Application No
PC:/IB 99/01430

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 C12N1/04 //(C12N1/20,C12R1:46)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 430 406 A (MEIJI MILK PROD CO LTD) 5 June 1991 (1991-06-05) cited in the application abstract	1-4
X	DATABASE WPI Section Ch, Week 9212 Derwent Publications Ltd., London, GB; Class B04, AN 92-092889 XP002105963 & JP 04 036180 A (MEIJI MILK PROD CO LTD), 6 February 1992 (1992-02-06) cited in the application abstract	1-4
A	US 2 966 445 A (ROLAND F. BEERS) 27 December 1960 (1960-12-27) the whole document	1-4

-/--

[X] Further documents are listed in the continuation of box C.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1999	15/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. ational Application No
PC./IB 99/01430

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0430406 A	05-06-1991	JP 2901008 B	02-06-1999
		JP 3219884 A	27-09-1991
		AU 634603 B	25-02-1993
		AU 6700290 A	06-06-1991
		CA 2029249 A	29-05-1991
		DE 69027098 D	27-06-1996
		NZ 236211 A	28-07-1992
		US 5075226 A	24-12-1991
JP 4036180 A	06-02-1992	NONE	
US 2966445 A	27-12-1960	NONE	